From: Murphy, Joseph P Maj USMC DARPA DIRO (USA) <[redacted]>
Sent:
To:
Cc:
Subject:

Capt xxxxx,

Thanks for responding.

I'm reaching out to communicate some information relative to COVID that I don't believe xxxxx or your director is aware of. You probably saw earlier this week that more official documents linking NIH and EcoHealth Alliance to the Wuhan Institute of Virology were published by The Intercept. I came across additional incriminating documents and produced an analysis shortly after leaving DARPA last month. This report was routed to the DOD IG office.

I'm unsure whether the significance of what I communicated is understood by those that received the report. Decisions with regards to the vaccines do not appear to be informed by analysis of the documents. The main points being that SARS-CoV-2 matches the SARS vaccine variants the NIH-EcoHealth program was making in Wuhan; that the DOD rejected the program proposal because vaccines would be ineffective and because the spike proteins being inserted into the variants were deemed too dangerous (gain-of-function); and that the DOD now mandates vaccines that copy the spike protein previously deemed too dangerous. To me, and to those who informed my analysis, this situation meets no-go or abort criteria with regards to the vaccines until the toxicity of the spike protein can be investigated. There's also information within the documents about which drugs effectively treat the program's SARS-CoVs.

Thus why I'm reaching out. I'm trying to help aid leadership grapple with the vaccines and the mandate with as much information as is available. I wanted to push this information your way.

Several of the documents referenced in the IG report have since been downgraded.

Please reach out to me with questions.

V/R,

Major Joe Murphy USMC
Marine Program Liaison
Code 34 & 35
Office of Naval Research
Work: [redacted]
Cell: [redacted]
[redacted]

DEFENSE ADVANCED RESEARCH PROJECTS AGENCY
675 NORTH RANDOLPH STREET
ARLINGTON, VA 22203-2114

13 Aug 21

From: COMMANDANT OF THE MARINE CORPS FELLOW, DARPA
To: INSPECTOR GENERAL

Subj: SARS-CoV-2 ORIGINS INVESTIGATION WITH US GOVERNMENT PROGRAM UNDISCLOSED DOCUMENT ANALYSIS

Ref: (1) Executive Slide HR00118Soo17 EcoHealth Alliance DEFUSE
(2) HR00118S0017-PREEMPT-FP-019-PM Summary (Selectable - Not Recommended)
(3) PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE
(4) PREEMPT Volume 2 EHA Final HR00118S0017 EcoHealth Alliance DEFUSE
(5) SF424_2_0-V2.0 HR00118S0017 EcoHealth Alliance DEFUSE
(6) WIV Budget packet HR001118S0017 EcoHealth Alliance DEFUSE
(7) WS00094394-RR_KeyPersonExpanded_2_0-V2.0 HR001118S0017 EcoHealth Alliance DEFUSE
(8) WS00094394-RR_PersonalData_1_2-V1.2 HR001118S0017 EcoHealth Alliance DEFUSE

1. SARS-CoV-2 is an American-created recombinant bat vaccine, or its precursor virus. It was created by an EcoHealth Alliance program at the Wuhan Institute of Virology (WIV), as suggested by the reporting surrounding the lab leak hypothesis. The details of this program have been concealed since the pandemic began. These details can be found in the EcoHealth Alliance proposal response to the DARPA[i] PREEMPT[ii] program Broad Agency Announcement (BAA) HR00118S0017, dated March 2018[iii] - a document not yet publicly disclosed.

The contents of the proposed program are extremely detailed. Peter Daszak lays out step-by-step what the organization intends to do by phase and by location. The primary scientists involved, their roles, and their institutions are indicated. The funding plan for the WIV work is its own document. The reasons why nonpharmaceutical interventions like masks and medical countermeasures like the mRNA vaccines do not work well can be extrapolated from the details. The reasons why the early treatment protocols work as curatives are apparent.

SARS-CoV-2's form as it emerged is likely as a precursor, deliberately virulent, humanized recombinant SARSr-CoV that was to be reverse engineered into a live attenuated SARSr-Cov bat vaccine. Its nature can be determined from analysis of its genome with the context provided by the EcoHealth Alliance proposal. Joining this analysis with US intelligence collections on Wuhan will aid this determination.

When synthesized with the EcoHealth Alliance proposal, US collections confirm EcoHealth Alliance was performing the work proposed. The analysts produce their reports in a vacuum, absent the context the proposal provides. As a fellow at DARPA, I could see both, and can do the synthesis. For instance, WIV personnel identified in intelligence reports are named in the proposal, these people use the lexicon of the proposal in the collections, and the virus variants proposed for experimentation are identical to those gleaned by collections. Moreover, I am also privy to information obtained by congressional office investigators and by DRASTIC[iv], which further corroborates that the program detailed in the BAA response was conducted until it was shut down in April 2020.

The purpose of the EcoHealth program, called DEFUSE[v] in the proposal, was to inoculate bats in the Yunnan, China caves where confirmed SARS-CoVs were found. Ostensibly, doing this would prevent another SARS-CoV pandemic; the bats' immune systems would be reinforced to prevent a deadly SARS-CoV from emerging. The specific language used is "inoculate bats with novel chimeric polyvalent spike proteins to enhance their adaptive immune memory against specific high-risk viruses."[vi] Being defense-related, it makes sense that EcoHealth submitted the proposal first to the Department of Defense, before it settled with NIH/NIAID. The BAA response is dated March 2018 and was submitted by Peter Daszak, president of EcoHealth Alliance.

DARPA rejected the proposal because the work was too close to violating the gain-of-function (GoF) moratorium,[vii] despite what Peter Daszak says in the proposal (that the work would not[viii]). As is known, Dr. Fauci with NIAID did not reject the proposal. The work took place at the WIV and at several sites in the US, identified in detail in the proposal.[ix]

The EcoHealth Alliance response to the PREEMPT BAA is placed along with other proposal documents in the PREEMPT folder on the DARPA Biological Technologies Office JWICS (top secret) share drive, address: Network/filer/BTO/CI Folder/PREEMPT

This folder was empty for a year. The files, completely unmarked with classification or distribution data, were placed in this folder in July 2021, which conspicuously aligns with media reporting, my probing, and Senator Paul's inquiry into NIH/NIAID gain-of-function programs. The unmarked nature combined with the timing signals that the documents were being hidden. No files at DARPA go unmarked in classification or distribution, including proprietary documents. Furthermore, PREEMPT is an unclassified program.

The files are also now held by Marine Corps Intelligence Activity (MCIA). They are identified in the reference block above.

2. SARS-CoV-2, hereafter referred to as SARSr-CoV-WIV, is a synthetic spike protein chimera engineered to attach to human ACE2 receptors and

inserted into a recombinant bat SARSr-CoV backbone. It is likely a live vaccine not yet engineered to a more attenuated state that the program sought to create with its final version. It leaked and spread rapidly because it was aerosolized so it could efficiently infect bats in caves, but it was not ready to infect bats yet, which is why it does not appear to infect bats. The reason the disease is so confusing is because it is less a virus than it is engineered spike proteins hitch-hiking a ride on a SARSr-CoV quasispecies swarm. The closer it is to the final live attenuated vaccine form, the more likely that it has been deattentuating since initial escape in August 2019.

The utility of certain countermeasures can be extrapolated from the documents:

- The team selected for SARSr-CoVs that were most monoclonal antibody and vaccine resistant.
- It is not practical to inoculate bats directly with shots, nor can bats get respiratory infections from droplets, so the team developed an aerosol to deliver the inoculations directly into the caves. To ensure it worked well, they developed the aerosol against *masked* civets.
- The proposal notes that interferon, Remdesivir, and chloroquine phosphate inhibit SARSr-CoV viral replication.

Because of its (now) known nature, the SARSr-CoV-WIV's illness is readily resolved with early treatment that inhibits the viral replication that spreads the spike proteins around the body (which induce a harmful overactive immune response as the body tries to clear the spikes from the ACE2 receptors). Many of the early treatment protocols ignored by the authorities work because they inhibit viral replication or modulate the immune response to the spike proteins, which makes sense within the context of what EcoHealth was creating. Some of these treatment protocols also inhibit the action of the engineered spike protein. For instance, Ivermectin (identified as curative in April 2020) works throughout all phases of illness because it both inhibits viral replication and modulates the immune response. Of note, chloroquine phosphate (Hydroxychloriquine, identified April 2020 as curative) is identified in the proposal as a SARSr-CoV inhibitor, as is interferon (identified May 2020 as curative).

The gene-encoded, or "mRNA," vaccines work poorly because they are synthetic replications of the already-synthetic SARSr-CoV-WIV spike proteins and possess no other epitopes. The mRNA instructs the cells to produce synthetic copies of the SARSr-CoV-WIV synthetic spike protein directly into the bloodstream, wherein they spread and produce the same ACE2 immune storm that the recombinant vaccine does. Many doctors in the country have identified that the symptoms of vaccine reactions mirror the symptoms of the disease, which corroborates with the similar synthetic nature and function of the respective spike proteins.
The vaccine recipient has no defense against the bloodstream entry, but their nose protects them from the recombinant spike protein quasispecies during "natural infection" (better termed as aerosolized inoculation).

Furthermore, the EcoHealth proposal states that a "vaccine approach lacks sufficient epitope coverage to protect against quasispecies of coronavirus."[x] Consequently, they were trying to make vaccines work by "targeted immune boosting via vaccine inoculators using chimeric polyvalent recombinant spike proteins."[xi] The nature of using a spike protein vaccine with one epitope against a spike protein vaccine with a quasispecies may explain the unusual (and potentially detrimental) antibody response amongst the vaccinated to the new COVID variants.[xii] Fundamentally, the knowledge the proposal provides signals that the risk of Antibody Dependent Enhancement (ADE) from vaccination should be evaluated with high priority, on top of the reality that single-epitope vaccines will have little effect against SARSr-CoV-WIV, as indicated in the proposal.

The potential for SARSr-CoV-WIV to deattentuate requires immediate attention. Live vaccines have been found to deattentuate in the past. If this is the case with SARSr-CoV-WIV, then the mass vaccination campaign actually performs an accelerated gain-of-function for it. Since it is designed for bats off of a human-susceptible SARS-CoV, vaccinating humans against it actually gains its function back towards a more deattenuated human-susceptible form. Improving the SARSr-CoV-WIV spike protein to gain robustness against monoclonal vaccines is one of the steps of the DEFUSE program. The mechanism to improve the SARSr-CoV-WIV spike protein (other than direct engineering) is to challenge it against animals that have spike protein-only antibodies. The attenuated virus will either die or adapt its form to neutralize the spike protein-only antibodies. The intent was to perform this task against humanized mice and then "batified" mice. Instead, it was done with the world's population.

SARSr-CoV-WIV is not meant to kill the bats, but to immunize them. This nature may explain its general harmlessness to most people, and its harmfulness to the old and comorbid, who are in general more susceptible to vaccine reactions. The asymptomatic nature is also explained by the bat vaccine-intention of its creators (a good vaccine does not generate symptoms). Such effects would be expected of an immature vaccine, or a vaccine being reverse engineered from a more virulent form into an attenuated form. The spike protein effect on ACE2 receptors exacerbates the harmfulness in accordance with age and comorbidity. The nature of SARSr-CoV-WIV's deattentuation will also indicate future virulence, though knowing its nature at last neutralizes the threat as effective treatments can be applied with confidence.

3. DRASTIC and other scientists will clean up my description of SARSr-CoV-WIV's nature and progression within the DEFUSE program. This information is sufficient for an investigative report and more than enough to correct the existing pandemic strategy. Previously, the nation did not know itself, nor the adversary in the pandemic conflict. Now it knows both. The problem can be framed appropriately and specifically against a confirmed hypothesis. Limiting disease transmission can be dropped as the implied strategic end, as it is not the actual problem,

nor is it actually feasible. The strategy will then align early treatment protocols and prophylaxis with the known curatives as ways and means. This course of action will achieve the strategic end of clinical resolution for those that are susceptible to the adverse effects from SARSr-CoV-WIV inoculation.

4. I will inevitably be asked how I figured this out and how I discovered the documents. The pandemic response became the predominant focus of my fellowship efforts. DARPA worked a number of pandemic innovations and much of its team was familiar with biodefense. I had the opportunity to "sit in the back row" per se and observe and listen-in on the government's efforts. My obligation-light fellowship also allowed me to observe and read the field. This observation grew in scope to the point that it became a series of reports, like a military scout would prepare when tasked to investigate a problem.

These reports served as iterative thinking against the problem over many months. Eventually, I arrived at a hypothesis that what leaked from the WIV could be a bat vaccine or its precursor. It was feasible that the US would try to avoid a SARS-CoV outbreak by stopping it at its source, not by halting its infections amongst people, but by halting the infections amongst the bats. Americans are creative, even if imprudent, and technologically confident enough to try it. This concept seemed to fit within the PREEMPT program construct as well, and DRASTIC had discovered that some earlier specimens within the USAID PREDICT program were obtained in Africa and sent to the WIV. Moreover, the unusual nature and pathology of the virus hinted that it could be a vaccine or be vaccine-like.

A technological challenge as difficult as inoculating bats in China would be tried at DARPA first. The massive, "Manhattan Project"-level of information suppression executed by the government and the Trusted News Initiative indicates that it would be covered-up if something bad happened. The lab-leak hypothesis and squabbling between Senator Paul and Dr. Fauci indicated that the cover up was more localized. Further, an actual cover-up would be more disciplined with its paperwork. So I presumed that unclassified files would be concealed on a higher network and found them where I expected them to be. I understood what they were and their content, pushed the files off-site, and compiled this report.

8/13/2021

X J. Murphy

Joseph Murphy
Major, US Marine Corps
Signed by: MURPHY.JOSEPH.PATRICK.1275023554

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[i] DARPA: Defense Advanced Research Projects Agency
[ii] PREEMPT: Preventing Emerging Pathogenic Threats

[iv] DRASTIC: Decentralized Radical Autonomous Search Team Investigating COVID-19. This collection of scientists and sleuths broke open the lab leak hypothesis into the mainstream and has picked apart Chinese and World Health Organization (WHO) reports on SARS-CoV-2's origins in Wuhan.
[v] DEFUSE: Defusing Threat of Bat-borne Coronavirus
[vi] PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE. Another description used: "We will develop recombinant chimera spike proteins from known SARSr-CoVs, and those characterized by DEFUSE, using details of SARS S protein structure and host cell binding, we will sequence, reconstruct, and characterize spike trimmers and RBDs of SARSr-CoVs, incorporate them into nanoparticles or raccoon poxvirus vectors for delivery to bats."
[vii] Dr. James Gimbert, DARPA Program Manager states: "team's approach does potentially involve GoF/DURC research (they aim to synthesize spike glycoproteins that may bind to human cell receptors and insert them into SARS-CoV backbones to assess capacity to cause SARS-like disease."
[viii] "We will commercially synthesize SARSr-CoV S glycoprotein genes, designed for insertion into SHC014 or WIV16 molecular clone backbones (88% and 97% S protein identity to epidemic SARS-Urbani). These are BSL-3, <u>not select agents or subject to P3CO</u>" (they use bat SARSr-CoV backbones which are exempt)"
[ix] Duke NUS Medical School, UNC, USGS National Wildlife Health Center, Palo Alto Rsesearch Center, Kumning, Signapore, and Madison, WI.
[x] PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE
[xi] PREEMPT Volume 1 no ESS HR00118S0017 EcoHealth Alliance DEFUSE
[xii] "For Delta, neutralizing antibodies have a decreased affinity for spike protein, while facilitating antibodies have a "strikingly increased" affinity for spike protein." Yahi, et al. "Infection-enhancing anti-SARS-CoV-2 antibodies recognize both the original Wuhan/D614G strain and Delta variants. A potential risk for mass vaccination?" *Journal of Infection*. August 9, 2021. https://www.journalofinfection.com/article/S0163-4453(21)00392-3/fulltext

DEFENSE ADVANCED RESEARCH PROJECTS AGENCY
675 NORTH RANDOLPH STREET
ARLINGTON, VA 22203-2114

PM SUMMARY SHEET
SOURCE SELECTION SENSITIVE

Solicitation Number: HR001118S0017
Solicitation Title: PREventing EMerging Pathogenic Threats (PREEMPT)
PM Name: James Gimlett
Proposer: EcoHealth Alliance
Proposal Title: Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses
Proposal Identifier: HR001118S0017-PREEMPT-FP-019

I have reviewed the attached proposal and Evaluation Reports and find that this proposal is selectable based on the evaluation criteria included in the BAA. However, I am not recommending funding at this time based on the rationale provided below.

Funding Requested (by proposer):

Phase I	Phase II	Total
$8,411,546	$5,797,699	$14,209,245

This proposal aims to identify and model spillover risk of novel, pandemic-potential SARS-related coronaviruses (SARSr-CoVs) in Asia, focusing specifically on known hotspot bat caves in China. In prior work under USAID Predict, the team identified high risk of SARSr-CoVs in specific caves in Asia. The project has a good running start since the hotspot caves already test positive, with high prevalence, for several SARSr viruses so the team won't be looking for needles in haystacks. The team will build on past surveillance work as well as some impressive work in developing geo-based risk maps of zoonotic hotspots based on past spillovers and ecological data. Two approaches are proposed to preempt zoonotic spillover through reduction of viral shedding in the bat caves: 1) innate immune boosting to downregulate viral regulation; 2) targeted immune boosting via vaccine innoculations using chimeric polyvalent recombinant spike proteins to protect against specific high risk viruses.

Two of three reviewers marked this proposal as Selectable. Key strengths are the experienced team and the selected coronavirus hotspot caves that show high prevalence for novel bat coronaviruses. Experimental in vivo and in vitro work is logically thought out and will be used to validate molecular and evolutionary models. Proposed preemption approaches, while somewhat conventional, have the advantage of a fast timeline for validation on bat or "batenized" mouse models. Multiple vaccine delivery mechanisms are proposed, including aerosolized spray, transdermal nanoparticle application, and edible adhesive gels. However, several weaknesses to the proposal were also noted. These include a lack of detail regarding data, statistical analyses and model development and how prior work will be leveraged and extended. Proposal also lacks clear decision points to assess the deployment and validation of TA2 preemption methods in the

SUMMARY OF PROPOSED COSTS

Wuhan Institute of Virology (WIV)
DARPA-BAA-HR001118S0017

		PHASE 1				PHASE 2				
		BASE 1 12/1/2018 Through 11/30/2019		BASE 2 12/1/2019 Through 11/30/2020		OPTION 1 12/1/2020 Through 11/30/2021		OPTION 2 12/1/2021 Through 5/31/2022		PROJECT TOTAL
Direct Labor - Senior and Key Personnel		37,975		37,975		37,975		22,153		136,078
Direct Labor - Other Personnel		37,027		40,824		40,824		18,987		137,662
Fringe Benefits		22,500		23,639		23,639		12,341		82,119
Total Direct Labor & Fringe Benefits		97,502		102,438		102,438		53,481		355,859
Materials and Supplies		167,661		198,167		210,687		56,597		643,113
Travel		16,739		7,282		15,523		8,027		47,571
Equipment		0		0		0		0		0
Other Direct Costs		8,200		6,200		6,200		8,200		28,800
Total Other Direct Costs		192,600		211,649		232,410		82,824		719,484
Subtotal: Direct Labor, Fringe, Overhead & Other Direct Co		290,102		314,087		334,848		136,305		1,075,343
Exclusion(s) From Base For F&A		0		0		0		0		0
Adjusted Base for F&A		290,102.25		314,087.15		334,848.25		136,305.25		1,075,342.90
F&A	10.0%	29,010.00	10.0%	31,409.00	10.0%	33,485.00	10.0%	13,631.00	10.0%	107,535.00
Total Proposed Cost		319,112.25		345,496.15		368,333.25		149,936.25		1,182,877.90

Wuhan Institute of Virology DARPA-BAA-HR001118S0017	DIRECT LABOR BREAKDOWN							
	PHASE ONE - BASE PERIOD (24 months)							
PROJECT DEFUSE	BASE 1			Total Salary Amount Y1	BASE 2			Total Salary Amount Y2
Personnel	Hourly Rate	# Months	# Hours		Hourly Rate	# Months	# Hours	
Investigator)	$25.56	3.00	528	$13,496	$25.56	3.00	528	$13,496
Dr. Peng Zhou (Senior	$18.26	6.00	1056	$19,280	$18.26	6.00	1056	$19,280
Dr. Ben Hu (Research Fellow)	$10.95	3.00	528	$5,784	$10.95	3.00	528	$5,784
Associate Professor	$13.69	6.00	1056	$14,460	$13.69	6.00	1056	$14,460
Senior Technician	$10.95	6.00	1056	$11,568	$10.95	6.00	1056	$11,568
Technician 1	$7.30	9.00	1584	$11,569	$7.30	6.00	1056	$7,712
Technician 2					$7.30	6.00	1056	$7,712
TOTAL DIRECT LABOR				$76,156				$80,012
		Rate	Base Amount	Total Fringe Y1		Rate	Base Amount	Total Fringe Y2
FRINGE BENEFITS		30.00%	$76,156.13	$22,846.84		30.00%	$80,012.31	$24,003.69
Fringe)				$99,002.97				$104,016.00

	PHASE TWO - OPTION PERIOD (18 months)							
	OPTION 1			Total Salary Amount Y3	OPTION 2			Total Salary Amount Y3.5
Personnel	Hourly Rate	# Months	# Hours		Hourly Rate	# Months	# Hours	
Dr. Zhengli Shi (Co-Investigator)	$25.56	3.00	528	$13,496	$25.56	2.00	352	$8,997
Dr. Peng Zhou (Senior Scientist)	$18.26	6.00	1056	$19,283	$18.26	3.00	528	$9,641
Dr. Ben Hu (Research Fellow)	$10.95	3.00	528	$5,782	$10.95	2.00	352	$3,854
Associate Professor	$13.69	6.00	1056	$14,457	$13.69	3.00	528	$7,228
Senior Technician	$10.95	6.00	1056	$11,563	$10.95	3.00	528	$5,782
Technician 1	$7.30	6.00	1056	$7,709	$7.30	3.00	528	$3,854
Technician 2	$7.30	6.00	1056	$7,709		3.00	528	$0
TOTAL DIRECT LABOR				$79,997				$39,357
		Rate	Base Amount			Rate	Base Amount	Total Fringe Y3.5
FRINGE BENEFITS		30.00%	$79,997.28	$23,999.18		30.00%	$39,357.12	$11,807.14
TOTAL LABOR (Salary + Fringe)				$103,996.46				$51,164.26

Total Labor	summary	e
$358,179.69	355,859	#######

WIV	Wuhan Institute of Virology - TRAVEL BREAKDOWN
DARPA BAA-HR001118S0017	

Total Travel: $47,571.00

Base 1	$16,739.00
Base 2	$7,282.00
Option 1	$15,523.00
Option 2	$8,027.00

Trip #: 1 — Y1 — Location: Arlington, VA — Contract Period: Base 1
Purpose: DARPA Kickoff Meeting (Shi +1 key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
4	2	$2,018.00	$69.00	$258.00	$212.00	$6,500.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$112.00
Transportation to/from Arlington airport	$100.00
Total:	$212.00

Trip #: 2 — Y1 — Location: New York, NY — Contract Period: Base 1
Purpose: Annual Meeting (Shi +2 key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
6	3	$949.00	$74.00	$291.00	$522.00	$9,935.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$168.00
Transportation to/from NYC airport	$354.00
Total:	$522.00

Trip #: 3 — Y3.5 — Location: Salt Lake City, UT — Contract Period: Base 2
Purpose: Ecological Society of America Conference (Baric + key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
4	3	$2,511.00	$59.00	$124.00	$196.00	$7,282.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$112.00
Transportation to/from SLC airport	84
Total:	$196.00

Trip #: 4 — Y3 — Location: New York, NY — Contract Period: Option 1
Purpose: Annual Meeting in NYC (Shi +2 key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
4	3	$949.00	$74.00	$291.00	$168.00	$7,395.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$168.00
Transportation to/from NYC airport	$354.00
Total:	$168.00

Trip #: 5 — Y2 — Location: Atlanta, GA — Contract Period: Option 1
Purpose: International Conference on Emerging Infectious Diseases (Shi +1 key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
4	2	$7,034.00	$69.00	$148.00	$224.00	$8,128.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$112.00
Transportation to/from Georgia airport	$112.00
Total:	$224.00

Trip #: 6 — Y3.5 — Location: New York, NY — Contract Period: Option 2
Purpose: Annual Meeting (Baric +2 key personnel)

Days	# of People	Airfare	Per Diem	Lodging	Other	Total
5	1	$949.00	$74.00	$291.00	$174.00	$2,948.00
4	2	$949.00	$74.00	$291.00	$261.00	$5,079.00

Itemized Expenses for "Other"

Description	Amount
Transportation to/from Wuhan airport	$168.00
Transportation to/from NYC airport	$354.00
Total:	$522.00

NOTE: Dr. SHI WILL STAY AN ADDITIONAL DAY FOR PROJECT CLOSEOUT

WIV

DARPA-BAA-HR001118S0017

PROJECT DEFUSE

Wuhan Institute of Virology - SUMMARY COST BUILDUP BY TASK

Task #	TECHNICAL AREA / TASK	PHASE 1		PHASE 2		TASK TOTAL
		Base 1	Base 2	Option 1	Option 2	
		12/1/18 - 11/30/19	12/1/19 - 11/30/20	12/1/20 - 11/30/21	12/1/21 - 5/31/22	
TA1-PI-T3.1	PCR screening of longitudinal specimens from target bat species	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T3.2	Genetically sequence SARSr-CoV spike proteins from PCR-positive samples	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.1	Design Luciferase Immunoprecipitation system (LIPS) assays to high- and low- jump risk SARSr-CoV QS0 we have characterized	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.2	Determine specificity of LIPS assays by recombinant protein or attenuated virus inoculation into rabbits	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.3	Validate LIPS assays using positive serum samples, spike protein based LIPS and viral neutralization	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.4	Test previously-collected human sera from Yunnan Province to assess SARSr-CoV QS spillover	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T7.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.1	Develop chimeric SARSr-CoV S immunogens	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.2	Design and test 2nd generation chimeric S glycoprotein immunogens in humanized mice	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.1	Identify specific sites (entry, exit points), identify FEA automatic aerosolization points, fine-tune deployment plan	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.2	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months before deployment proof-of-concept experiment (EHA Consultant Zhu, WIV)	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.3	Run deployment experiment of most effective immune boosting molecules and delivery techniques via FEA aerosolization mechanism at one test and two control bat cave sites in Yunnan, China	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.4	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months after deployment proof-of-concept experiment	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.5	Assess efficacy of proof-of-concept trial.	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
						$ 1,182,877.90

WIV DARPA-BAA- HR001118S0017

	SUMMARY COST BUILDUP BY PHASE					
	Phase I: 24 MONTHS 12/1/18 - 11/30/20		Phase II: 18 MONTHS 12/1/20 - 05/30/22		All Phases 42 MONTHS 12/1/18 - 5/30/22	
Personnel	$	153,801	$	119,939	$	273,740
Fringe Benefits	$	46,139	$	35,980	$	82,119
Supplies	$	365,828	$	277,285	$	643,113
Travel	$	24,021	$	23,550	$	47,571
Other Direct Costs	$	14,400	$	14,400	$	28,800
Indirect Costs	$	60,419	$	47,116	$	107,535
TOTAL	$	664,608	$	518,270	$	1,182,878

	SUMMARY COST BUILDUP BY YEAR									
	Year 1		Year 2		Year 3		Year 4		TOTAL PROJECT	
Personnel	$	75,002	$	78,799	$	78,799	$	41,140	$	273,740
Fringe Benefits	$	22,500	$	23,639	$	23,639	$	12,341	$	82,119
Supplies	$	167,661	$	198,167	$	210,687	$	66,597	$	643,113
Travel	$	16,739	$	7,282	$	15,523	$	8,027	$	47,571
Other Direct Costs	$	8,200	$	6,200	$	6,200	$	8,200	$	28,800
Indirect Costs	$	29,010	$	31,409	$	33,485	$	13,631	$	107,535
TOTAL	$	319,112	$	345,496	$	368,333	$	149,936	$	1,182,878

	WIV DARPA-BAA-HR001118S0017 PROJECT DEFUSE	Wuhan Institute of Virology - SUMMARY COST BUILDUP BY TASK				
		PHASE 1		PHASE 2		
Task #	TECHNICAL AREA / TASK	Base 1 12/1/18 - 11/30/19	Base 2 12/1/19 - 11/30/20	Option 1 2/1/20 - 11/30/2	Option 2 12/1/21 - 5/31/22	TASK TOTAL
TA1-PI-T3.1	PCR screening of longitudinal specimens from target bat species	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T3.2	Genetically sequence SARSr-CoV spike proteins from PCR-positive samples	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.1	Design Luciferase Immunoprecipitation system (LIPS) assays to high- and low-jump risk SARSr-CoV QS0 we have characterized	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.2	Determine specificity of LIPS assays by recombinant protein or attenuated virus inoculation into rabbits	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.3	Validate LIPS assays using positive serum samples, spike protein based LIPS and viral neutralization	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA1-PI-T6.4	Test previously-collected human sera from Yunnan Province to assess SARSr-CoV QS spillover	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T7.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.1	Develop chimeric SARSr-CoV S immunogens	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.2	Design and test 2nd generation chimeric S glycoprotein immunogens in humanized mice	$ 35,456.92	$ 38,388.46	$ -	$ -	$ 73,845.38
TA2-PI-T8.5	Test targeted immune boosting in wild-caught captive Rhinolophus spp.	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.1	Identify specific sites (entry, exit points), identify FEA automatic aerosolization points, fine-tune deployment plan	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.2	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months before deployment proof-of-concept experiment (EHA Consultant Zhu, WIV)	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.3	Run deployment experiment of most effective immune boosting molecules and delivery techniques via FEA aerosolization mechanism at one test and two control bat cave sites in Yunnan, China	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.4	Conduct bat viral surveillance of one test-site cave and two control caves at our cave complex to assess baseline data for 4 months after deployment proof-of-concept experiment	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
TA2-P2-T5.5	Assess efficacy of proof-of-concept trial.	$ -	$ -	$ 61,388.88	$ 24,989.38	$ 86,378.25
						$ 1,182,877.90

Wuhan Institute of Virology DARPA-BAA-HR001118S0017	DIRECT LABOR BREAKDOWN							
	PHASE ONE - BASE PERIOD (24 months)							
PROJECT DEFUSE	BASE 1			Total Salary Amount Y1	BASE 2			Total Salary Amount Y2
Personnel	Hourly Rate	# Months	# Hours		Hourly Rate	# Months	# Hours	
Investigator)	$25.56	3.00	528	$13,496	$25.56	3.00	528	$13,496
Dr. Peng Zhou (Senior	$18.26	6.00	1056	$19,280	$18.26	6.00	1056	$19,280
Dr. Ben Hu (Research Fellow)	$10.95	3.00	528	$5,784	$10.95	3.00	528	$5,784
Associate Professor	$13.69	6.00	1056	$14,460	$13.69	6.00	1056	$14,460
Senior Technician	$10.95	6.00	1056	$11,568	$10.95	6.00	1056	$11,568
Technician 1	$7.30	9.00	1584	$11,569	$7.30	6.00	1056	$7,712
Technician 2					$7.30	6.00	1056	$7,712
TOTAL DIRECT LABOR				$76,156				$80,012
		Rate	Base Amount	Total Fringe Y1		Rate	Base Amount	Total Fringe Y2
FRINGE BENEFITS		30.00%	$76,156.13	$22,846.84		30.00%	$80,012.31	$24,003.69
Fringe)				$99,002.97				$104,016.00

	PHASE TWO - OPTION PERIOD (18 months)							
	OPTION 1			Total Salary Amount Y3	OPTION 2			Total Salary Amount Y3.5
Personnel	Hourly Rate	# Months	# Hours		Hourly Rate	# Months	# Hours	
Dr. Zhengli Shi (Co-Investigator)	$25.56	3.00	528	$13,496	$25.56	2.00	352	$8,997
Dr. Peng Zhou (Senior Scientist)	$18.26	6.00	1056	$19,283	$18.26	3.00	528	$9,641
Dr. Ben Hu (Research Fellow)	$10.95	3.00	528	$5,782	$10.95	2.00	352	$3,854
Associate Professor	$13.69	6.00	1056	$14,457	$13.69	3.00	528	$7,228
Senior Technician	$10.95	6.00	1056	$11,563	$10.95	3.00	528	$5,782
Technician 1	$7.30	6.00	1056	$7,709	$7.30	3.00	528	$3,854
Technician 2	$7.30	6.00	1056	$7,709		3.00	528	$0
TOTAL DIRECT LABOR				$79,997				$39,357
		Rate	Base Amount			Rate	Base Amount	Total Fringe Y3.5
FRINGE BENEFITS		30.00%	$79,997.28	$23,999.18		30.00%	$39,357.12	$11,807.14
TOTAL LABOR (Salary + Fringe)				$103,996.46				$51,164.26

Total Labor	summary	e
$358,179.69	355,859	#######

Project DEFUSE: Defusing the Threat of Bat-borne Coronaviruses



Principal Investigator and Technical Point of Contact	Administrative Point of Contact
Peter Daszak, Ph.D.	Luke Hamel
EcoHealth Alliance	EcoHealth Alliance
460 West 34th Street, 17th Floor	460 West 34th Street, 17th Floor
New York, NY 10001	New York, NY 10001
(p) 212-380-4474	(p) 646-868-4709
(e) daszak@ecohealthalliance.org	(e) hamel@ecohealthalliance.org
(f) 212-380-4465	(f) 212-380-4465

Identifying Number: HR001118S0017-PREEMPT-PA-001
Award Instrument Requested: Grant
Places and Periods of Performance: 12/1/18 - 5/31/22; Palo Alto, CA; Kunming and Wuhan, China; Chapel Hill, NC; New York, NY; Singapore; Madison, WI
Total funds requested: $14,209,245
Proposal validity period: 6 months
Date proposal submitted: 3/27/18

24 March 2018

Dear Committee for DARPA PREventing EMerging Pathogenic Threats (PREEMPT),

Please accept the following proposal to the PREventing EMerging Pathogenic Threats (PREEMPT, HR001118S0017) program. The PI for this project is:

Dr. Peter Daszak
President, EcoHealth Alliance
460 W. 34th Street, 17th Floor
New York, NY 10001
212-380-4474

Title: Project Defuse: Defusing the Threat of Bat-Borne Coronaviruses

Amount of the Requested Proposal: $14,209,245

Thank you for your time, and I look forward to hearing from you. If you have any questions, do not hesitate to call or email me.

Yours sincerely,

Aleksei Chmura
Chief of Staff, EcoHealth Alliance
460 W. 34th Street, 17th Floor
New York, NY 10001
212-380-4473

Section II

A. EXECUTIVE SUMMARY

Technical Approach: Our goal is to defuse the potential for spillover of novel bat-origin high-zoonotic risk SARS-related coronaviruses in Asia. In TA1 we will intensively sample bats at our field sites where we have identified high spillover risk SARSr-CoVs. We will sequence their spike proteins, reverse engineer them to conduct binding assays, and insert them into bat SARSr-CoV (WIV1, SHC014) backbones (these use bat-SARSr-CoV backbones, not SARS-CoV, and are exempt from dual-use and gain of function concerns) to infect humanized mice and assess capacity to cause SARS-like disease. Our modeling team will use these data to build machine-learning genotype-phenotype models of viral evolution and spillover risk. We will uniquely validate these with serology from previously-collected human samples via LIPS assays that assess which spike proteins allow spillover into people. We will build host-pathogen spatial models to predict the bat species composition of caves across Southeast Asia, parameterized with a full inventory of host-virus distribution at our field test sites, three caves in Yunnan Province, China, and a series of unique global datasets on bat host-viral relationships. By the end of Y1, we will create a prototype app for the warfighter that identifies the likelihood of bats harboring dangerous viral pathogens at any site across Asia.

In TA2, we will evaluate two approaches to reduce SARSr-CoV shedding in cave bats: (1) Broadscale immune boosting, in which we will inoculate bats with immune modulators to upregulate their innate immune response and downregulate viral replication; (2) Targeted immune boosting, in which we will inoculate bats with novel chimeric polyvalent recombinant spike proteins plus the immune modulator to enhance innate immunity against specific, high-risk viruses. We will trial inoculum delivery methods on captive bats including a novel automated aerosolization system, transdermal nanoparticle application and edible adhesive gels. We will use stochastic simulation modeling informed by field and experimental data to characterize viral dynamics in our cave test sites, maximize timing, inoculation protocol, delivery method and efficacy of viral suppression. The most effective biologicals will be trialed in our test cave sites in Yunnan Province, with reduction in viral shedding as proof-of-concept.

Management Approach: Members of our collaborative group have worked together on bats and their viruses for over 15 years. The lead organization, EcoHealth Alliance, will oversee all work. EHA staff will develop models to evaluate the probability of specific SARS-related CoV spillover, and identify the most effective strategy for delivery of both immune boosting and immune targeting inocula. Specific work will be subcontracted to the following organizations:

- Prof. Baric, Univ. N. Carolina, will lead targeted immune boosting work, building on his two-decade track record of reverse-engineering CoV and other virus spike proteins.
- Prof. Wang, Duke-Natl. Univ. Singapore, will lead work on broadscale immune boosting, building on his group's pioneering work on bat immunity.
- Dr. Shi, Wuhan Institute of Virology will conduct viral testing on all collected samples, binding assays and some humanized mouse work.
- Dr. Rocke, USGS National Wildlife Health Center will optimize delivery of immune modulating biologicals, building on her vaccine delivery work in wildlife, including bats.
- Dr. Unidad, Palo Alto Research Center will lead development of novel delivery automated aerosolization mechanism for immune boosting molecules.

We are requesting $14,209,245 total funds for this project across 3.5 project years.

Section II

C. GOALS AND IMPACT

Overview: The overarching goals of DEFUSE are to:

- Identify and model spillover risk of novel SARS-related coronaviruses (SARSr-CoVs) in Asia.
- Design and demonstrate proof-of-concept that upregulating the naturally low innate immunity of bats (broadscale immune boosting) and targeting high risk SARSr-CoVs in particular (targeted immune boosting) will transiently reduce spillover risk.

Our strategy to reduce risk of viral emergence from bats will protect the warfighter within USPACOM, and will be scalable to other regions and viruses (Ebola, Henipaviruses, rabies).

Innovation and uniqueness:



Bats harbor more emerging zoonoses than any other group of mammals, are ubiquitous, abundant, and often overlooked. However, other than PPE, there is no available technology to reduce exposure risk to novel CoVs from bats, and no effective therapeutics or countermeasures. SARSr-CoVs are enzootic in Asian[1-3], African[4], and European bats[5,6] that roost in caves but forage widely at night, shedding virus in their feces and urine. We have now published direct evidence of spillover of novel SARSr-CoVs into people in Yunnan Province, China, close to a cave complex where we have isolated strains that produce SARS-like disease in humanized mice but don't respond to antibody treatment or vaccination. These viruses are a clear-and-present danger to our military and to global health security because of their circulation and evolution in bats and periodic spillover into humans.

EcoHealth Alliance (EHA) leads the world in predictive models of viral emergence. We will use machine-learning models of spillover hotspots, host-pathogen spatial and genotype-phenotype mapping, and unique datasets to validate and refine hotspot risk maps of viral emergence. We have shown that dampened innate immunity in bats allows them to carry otherwise lethal viruses, likely as an adaptation to the physiologic stress of flight. We will design strategies like small molecule RIG-like receptor (RLR) or Toll-like receptor (TLR) agonists, to upregulate bat immunity, and suppress viral replication, thereby significantly reducing viral shedding and spillover (broadscale immune boosting). We will complement this by coupling agonist treatments with SARSr-CoV recombinant spike proteins to boost pre-existing adaptive immune response adult bats against specific, high-risk SARSr-CoVs (targeted immune boosting). We will design novel delivery and automated application methods, based on our previous work on wildlife vaccines, to reduce hazard during deployment.

Technical Area 1



Our strategy begins by a complete inventory of bats and their SARSr-CoVs at our intervention test site cave complex in Yunnan, China that harbors bats with high-risk SARSr-CoVs. We will collect data from three caves in that system (one is our intervention test site and two control sites) on: monthly bat abundance and diversity, viral prevalence and diversity, individual bat viral load and host physiological markers; genomic characterization of low- and high-risk SARSr-CoV strains among bat species, sexes, and age classes; satellite telemetry and mark-recapture data on bat home range and inter-cave movement; and monitoring of daily, weekly and seasonal changes in bat populations. We will use stochastic neural networks to build joint species distribution models (JSDM) to predict bat species composition of caves, and high-risk SARSr-CoV diversity across S. China, South and SE Asia. These will be parameterized with EHA's database of bat host-viral relationships and estimates of zoonotic viral richness per bat species[7]; biological inventory data on all bat caves in Southern China; the full SARSr-CoV inventory from our cave test sites in Yunnan; and species distribution data for all bats. We will test and validate viral diversity predictions using data from >10,000 previously collected bat samples from 6 Asian countries under our USAID-funded PREDICT project. We will produce a prototype app for the warfighter to identify the risk of bats harboring dangerous viruses at a site. This 'spatial viral spillover risk' app will be field-deployable and updated real-time with surveillance data, to ground-truth and fine-tune predictions.

To characterize spillover risk of SARSr-CoV quasispecies (QS), the Wuhan Institute of Virology team (WIV) will test bat fecal, oral, and blood samples for SARSr-CoVs by PCR. We will collect viral load data from fresh fecal pellets. SARSr-CoV spike proteins will be sequenced, viral recombination events identified, and isolates used to identify strains that can replicate in human cells. The Univ. N. Carolina (UNC) team will reverse-engineer spike proteins of a large sample of high- and low-risk viruses for further characterization. This will effectively freeze the QS we analyze at t=0. These QS_0 strain viral spike glycoproteins will be synthesized, and those binding to human cell receptor ACE2 will be inserted into SARSr-CoV backbones (non-DURC, non-GoF), and inoculated into humanized mice to assess capacity to cause SARS-like disease, efficacy of monoclonal therapies, the inhibitor GS-5734[8] or vaccines against SARS-CoV[8-12].

We will use these data to build machine-learning genotype-to-phenotype Bayesian network models of viral evolution and host jump risk. These will predict the capacity of QS_0 strains to infect human cells based on genetic traits and experimental assays above. Using data on diversity of spike proteins, recombinant CoVs, and flow of genes via bat movement and migration, we will estimate evolutionary rates, rates of recombination, and capacity to generate novel strains capable of human infection. Finally, virus-host relationship and bat home range data will be used to estimate spillover potential - extending models well beyond our field sites. We will validate model predictions of host jump risk by 1) conducting further spike

protein-based binding and cell culture experiments, and 2) identifying whether designated high-risk SARSr-CoV strains have already spilled over into people near our bat cave sites. Our preliminary work shows ~3% seroprevalence to bat SARSr-CoVs in people at this site[13]. We will test these previously collected human sera (n>2000) for presence of antibodies to the high- and low-risk SARSr-CoVs identified by our modeling, using Luciferase immunoprecipitation system (LIPS) assays we design against the SARSr-CoVs identified in this project[14].

Technical Area 2

In TA2, we will develop scalable approaches to suppress SARSr-CoVs within bat reservoir species, to reduce the likelihood of virus transmission into humans. We will evaluate two approaches to defuse spillover potential: 1) **Broadscale immune boosting:** we will apply immune modulators like bat interferon and TLR agonists to up-regulate bat innate immunity and suppress viral replication and shedding; 2) **Targeted immune boosting:** we will apply polyvalent chimeric recombinant SARSr-CoV spike proteins in the presence of broadscale immune boosting treatments to boost immune memory and suppress specific SARSr-CoVs.

Both TA2 lines of work will run parallel beginning Yr 1. Prof. Wang (Duke-Natl. Univ. Singapore – Duke-NUS) will lead the broadscale immune boosting work, building on his pioneering work on bat immunity[15], including identifying weakened functionality of innate immunity factors like STING, a central DNA-interferon (IFN) sensing molecule, that may allow bats to maintain an effective, but not over-response to viruses[16], and IFNA, which is constitutively expressed without stimulation[17]. We will trial the following, concurrently and competitively, for efficacy and scalability: i) Activating TLR/RLR pathways to induce IFN induction, e.g. polyIC or 5'ppp-dsRNA. A similar strategy has been demonstrated in a mouse model for SARS-CoV[18,19]; ii) Universal bat interferon. Interferon has been used clinically in people, e.g. against filoviruses[20], and replication of SARSr-CoV is sensitive to interferon[21]; iii) Boosting bat IFN by blocking negative regulators. Bat IFNα is constitutively expressed but cannot be induced to a high level[17]. We will use CRISPRi to identify potential negative regulators and screen for compounds targeting this gene; iv) Activating dampened IFN production pathways via DNA-STING-dependent and ssRNA-TLR7-dependent pathways. Mutant bat STING restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence[16]. We will directly activate the pathways downstream of STING/TLR7, to promote viral clearance; v) inoculating crude CoV fragments to upregulate innate immune responses to specific CoVs – a partial step towards the targeted immune boosting work below.

Prof. Baric (UNC) will lead the targeted immune boosting work. We will develop recombinant chimeric spike-proteins[22] from known SARSr-CoVs, and those characterized by DEFUSE. Using details of SARS S protein structure and host cell binding[23] we will sequence, reconstruct and characterize spike trimers and receptor binding domains of SARSr-CoVs, incorporate them into nanoparticles or raccoon poxvirus-vectors for delivery to bats[10,24-27]. In combination with immune-boosting small molecules, we will use these to boost immune memory in adult bats previously exposed to SARSr-CoVs, taking the best candidate forward for field-testing. Recombinant S glycoprotein-based constructs with immunogenic blocks from across group 2B SARSr-CoVs should induce broadscale adaptive immune responses that reduce heterogeneous virus burdens in bats and transmission risk to people[28,29]. Innate immune damping is highly conserved in all bat species tested so far. We will use the unique Duke-NUS

Asian cave bat (*Eonycteris spelaea*) breeding colony to conduct initial proof-of-concept tests, extended to small groups of wild-caught *Rhinolophus sinicus* bats at WIV.

A novel delivery method for our immune boosting molecules will be developed and implemented by Dr. Rocke at the USGS National Wildlife Health Center (NWHC) who has previously developed animal vaccines through to licensure[10]. Using locally acquired insectivorous bats[11,12], we will assess delivery vehicles and methods including: 1) transdermally applied nanoparticles; 2) sticky edible gels that bats mutually groom and consume; 3) aerosolization via prototype sprayers (Dr. Unidad, PARC) designed for cave settings; and 4) automated sprays triggered by timers and movement detectors at critical cave entry points We have extensive preliminary data on these techniques for wildlife, including vaccinating bats against rabiesin the lab[11], successful delivery, consumption and spread in wild vampire bats. We will use the NWHC captive bat colony and wild bats in US caves to trial delivery vehicles using the biomarker rhodamine B (which fluorescently marks hair on consumption) to assess uptake. The most optimal deployment approaches will be tested on wild bats at our test cave sites in Yunnan, using the most effective immune modulation preparations. Bat populations from experimental and control caves will be surveyed longitudinally for viral load before and after deployment trials. EHA has had unique access to these sites for ~10 years. In DEFUSE Yr1, we will seek permission for experimental trials from collaborators at the Yunnan Forestry Department and Center for Disease Control, following our proven track record of rapidly obtaining IACUC and DoD ACURO approval for animal research. We will model optimal strategies to maximize treatment efficacy for TA2, using stochastic simulation modeling of viral circulation dynamics at our sites, informed by field and experimental data. We will estimate frequency and population coverage required for our intervention, and model the time period of viral suppression, until re-colonization or evolution leads to return of a high-risk SARSr-CoV.

Deliverables:

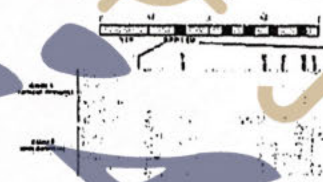
- Open source models and App identifying geographical and host-specific risk of spillover for novel SARSr-CoVs
- Experimentally validated genotype-phenotype models of spillover for viral strains.
- Proven technology to modulating bat innate immunity to reduce viral shedding
- Tested and validated delivery mechanism for bat cave usage including vaccines in other bat host-pathogen systems (e.g. rabies, WNS).
- Proof-of-concept approach to transiently reducing viral shedding in wild bats that can be adapted for other systems including Ebola virus.

Section II: D. TECHNICAL PLAN

Technical Area I:

Choice of site and model host-virus system: For the past 14 years, our team has conducted CoV surveillance in bat populations across S. China, resulting in >180 unique SARSr-CoVs in ~10,000 samples (>5% prevalence, including multiple individuals harboring the same viral strains)[2,21,33] and a per-bat species prevalence up to 10.9%. Bat SARSr-CoVs are genetically diverse, especially in the S gene, and most are highly divergent from SARS-CoV. However, our test cave site in Yunnan Province, harbors a quasispecies (QS) population assemblage that contains all the genetic components of epidemic SARS-CoV[34]. We have isolated three strains there (WIV1,

WIV16 and SHC014) that unlike other SARSr-CoVs, do not contain two deletions in the receptor-binding domain (RBD) of the spike, have far higher sequence identity to SARS-CoV (Fig. 1), use human ACE2 receptor for cell entry, as SARS-CoV does (Fig. 2), and replicate efficiently in various animal and human cells[2,8,33-35], including primary human lung airway cells, similar to epidemic SARS-CoV[11,12]. Chimeras (recombinants) with these SARSr-CoV S genes inserted into a SARS-CoV backbone, and synthetically reconstructed full length SHC014 and WIV1 cause SARS-like illness in humanized mice (mice expressing human ACE2), with clinical signs that are not reduced by SARS-CoV monoclonal antibody therapy or vaccination[11,12]. People living up to 6 kilometers from our test cave have SARSr-CoV antibodies (~3% seroprevalence)[13], suggesting active spillover. These data, phylogeography of SARSr-CoVs, and coevoutionary analysis of bats and their CoVs (unpubl.), suggest that bat caves in SW China, and *Rhinolophus* spp. bats are the likely origin of the SARS-CoV clade, and are a clear-and-present danger for the emergence of a SARSr-CoV from the current QS. The *Rhinolophus* spp. bats that harbor these viruses occur across Asia, Europe, and Africa. Thus, while DEFUSE fieldwork will focus on high-risk sites in S. China, our approach to reduce the risk of these viruses spilling over is broadly applicable across four combatant command regions (PACOM, CENTCOM, EUCOM, AFRICOM).



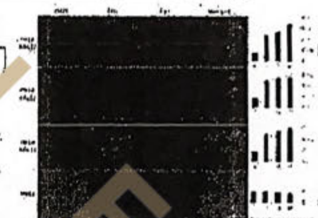


Fig. 1 (top left): Alignment of amino acid sequences of the spike protein receptor-binding motif of SARSr-CoVs and SARS-CoV[34]. Numbered amino acid residues are responsible for interaction with human ACE2[36]. Fig. 2 (top right): Bat SARSr-CoV WIV1 replicates efficiently in HeLa cells expressing human, civet and bat ACE2[2].

Full Inventory of bat SARSr-CoV QS at our test cave sites, Yunnan, China. To provide data to train and validate our modeling, and as baseline for our immune modulation trial (TA2), DEFUSE fieldwork will target the high-risk cave site in Yunnan Province, SW China (Fig. 4, red triangle) where we will conduct our field trial, and where we have previously identified and isolated high-risk SARSr-CoVs[2,11,33,34]. At three cave sites (one designated for our trial, two as controls), we will determine the baseline QS_0 risk of SARSr-CoV spillover. We will conduct longitudinal surveillance of bat populations to detect and isolate SARSr-CoVs, determine changes in viral prevalence over time, and measure bat population demographics and movement, definitively characterizing their SARSr-CoV host-viral dynamics. Field data will allow us to test the accuracy of our model predictions and compare efficacy of lab animal models with field trials. Our preliminary data (Table 1) demonstrate that *R. sinicus*, *R. ferrumequinum*, and *R. affinis* (which co-roost at our test site) are primary reservoirs of SARSr-CoV and the only reservoirs of three high-risk strains (WIV1, WIV16, SHC014), with *Hipposideros* and *Myotis* spp. playing an

insignificant (<1% prevalence) role in viral dynamics. We will capture *Rhinolophus spp.* bats using harp traps and mist nets during evening flyout, collect rectal, oral, and whole blood samples (×2 per bat) using sterile technique to avoid cross-contamination, and take 2-mm wing tissue punch biopsies for host DNA bar-coding, host ACE2 receptor gene sequencing (interface site – 3 individuals per species), and cophylogeny analyses. Bats will be subcutaneously microchipped (PIT tag), and morphological and physiological data (age class, body weight, reproductive status etc.).

Species	n	SARSr-CoV prevalence
Rhinolophus sinicus	1036	10.9%
R. ferrumequinum	191	6.3%
R. affinius	518	1.2%

In Phase I we will sample 60 bats each of *R. sinicus*, *R. ferrumequinum*, and *R. affinis*, (180 bats per cave) every three months non-destructively for 18 months from our three cave sites. Given ~6-9% prevalence (n=3,304) of SARSr-CoVs in *Rhinolophus* spp. at our sites, this sample size would allow detection of 10% fluctuation in viral prevalence among sampling periods and caves. During the 2 months per quarter without physical bat trapping we will collect fresh fecal pellets by placing clean $2m^2$ polyethylene sheets beneath roosting bats[37]. *Rhinolophus* spp. have a 7-week gestation period, spring birthing, and aggregate during mating periods. Our monthly sampling strategy will collect adequate data to parameterize stochastic simulation models, and cover two mating and gestation periods to assess life-history driven changes in viral prevalence and immune marker (e.g. interferon) levels. We will conduct pre- and post-intervention sampling (biweekly fecal pellet sampling for 4 months, and 10 male and 10 female bats per species tested every 2 weeks post-intervention for 4 months, prior to- and post-deployment) to monitor SARSr-CoV QS and bat immune status changes in test and control site bats during Phase II (TA2). Immune status can be followed in individual bats due to the relatively small roost sizes in these caves and our individual marking of captured bats. We will be assess immune status using nanostring immune profiling panels validated during captive bat studies at Duke NUS. We will use infrared spotlights and digital infrared imaging to record the number and species of bats above each plastic sheet and fecal pellets will be genetically barcoded to confirm species identification[18]. Samples will be preserved in viral transport medium, immediately frozen in liquid nitrogen dry shippers, and transported to partner laboratories with maintained cold chain and under strict biosafety protocols. PIT tag readers and weatherproof thermal imaging IR cameras mounted at each cave entrance will passively monitor temporal roost site fidelity, rates of inter-cave movement, and daily fluctuation in bat population[39]. ICARUS satellite transmitters (1g) will be attached to 12 *Rhinolophus* spp. bats from each study roost (36 bats total) to determine nightly foraging dispersal patterns (https://icarusinitiative.org). Telemetry and PIT tag data will be used to calculate home range, degree of mixing among roosts, and parameterize dynamic models. Study caves will be surveyed using portable LIDAR technology[40-42], to give a 3-D image of roost areas and data on species composition for targeting of immune modulation treatments in TA2 (Fig. 3). Sampling quotas will be adjusted based on lab and model results to optimize viral detection.



Fig. 3: Light Detection and Ranging (LIDAR) scanning to characterize caves and quantify number of individual bats roosting in clusters: A) LIDAR system takes a 360° omnidirectional photo of clustered bats, B) photo converted to 3-D point cloud, C) non-bat points, based on laser return intensity removed, D) automated counting algorithm counts individual bats. Figure from[41].

Our team has more than 50 years collective experience in safe and humane handling of bats for biological sampling. This project will operate under appropriate IACUC/ACURO and PPE guidelines. EHA has several ongoing DTRA-supported projects, has obtained ACURO approval for animal research from the DoD, and currently maintains IACUC protocols through Tufts University (EHA staff are adjunct faculty), which we will use for DEFUSE IACUCs. IACUCs already approved for lab/field work at Duke-NUS, UNC, NWHC, and WIV, will be modified for DEFUSE.

Predictive models of high-risk sites and bat species across Asia. We will build models that predict bat and viral diversity and spillover risk across Asia to enable warfighters and planners to assess risk and necessity for intervention deployment (TA2). We will combine regional-scale joint species distribution models (JSDM), machine-learning host-virus association models, and non-parametric viral richness estimators to respectively predict the composition of bat communities in caves across Asia, host range for key viral clades, and as-of-yet unsampled viral diversity. We will use a stochastic feedforward neural network to implement JSDMs that are effective at multiple scales with incomplete observations (as occurs for bats and their viruses), and that account for bat species co-occurrence driven by environment or evolution[43]. We will fit our JSDM to biological inventory data on over 200 caves in the region[44], to physiologically relevant bioclimatic variables (BIOCLIM)[45], open source topographic data, and proxies for subterranean habitat such as ruggedness and habitat heterogeneity. As in previous work[46], we will refine these models with regional-scale environmental variables (land-use, distance to roads, etc.) and cave-specific variables (cave length, availability of roosting area, entrance dimensions, cave complexity etc.). We will validate them using independent bat occurrence estimates and observations[47,48], and use EHA's unique database of all known host-virus relationships to extend predictions of bat CoV diversity and host range[7] (Fig. 4). We will use generalized additive host trait predictive models and machine-learning algorithms (BRT, random forest)[49] with non-parametric estimators to predict SARSr-CoV diversity in the QS of each bat species[50], and assess viral discovery rates in real time through sampling (Fig. 5).

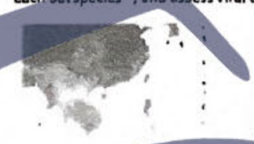


Fig. 4: Predictive map of zoonotic viral diversity in bats for China and SE Asia (yellow=more viruses), based on all known mammal host-viral relationships[7]. Our Yunnan test cave site is labeled (red asterisk). Fig. 5: CoV QS diversity estimates (dashed line with 95% confidence intervals) based on PREDICT sampling data (solid line) for four bat genera.

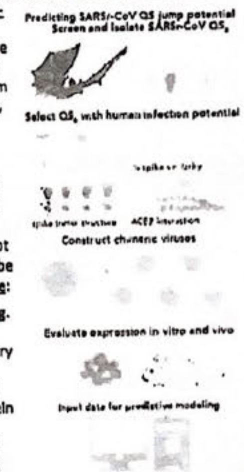
To extend the geographic scope of predictive models, we will include data from >1800 viral detections (CoVs and others) from >10,000 individual bat samples in 6 Asian countries (NIAID- and USAID PREDICT-funded). For species composition and viral presence predictions, we will validate models against a 20% validation subset of data, and field data.

Prototype app for the warfighter. Drawing on experience building applications for data collection and analysis for DoD (e.g. [illegible], [illegible], [illegible]), we will produce a prototype 'spatial viral spillover risk' app for the

warfighter that identifies probability of dangerous viral pathogens spilling over from bats at a site. We will use outputs from our spatial risk modeling, observed and predicted host-viral associations, open-source species and pathogen ontologies, and app-directed crowd-sourced echolocation data to ground-truth and fine-tune its predictive capacity. This app will be updated in Y2 and Y3 to incorporate additional risk data from host-virus binding assays and SARSr-CoV surveys. We will use EHA's risk-ranking algorithms ([illegible]) to display critical areas of high risk based on geolocation features, recency of information, host and pathogen characteristics. The app will collect user GPS location data and preload bat species distribution and community composition estimates from our JSDMs. These will be refined with real-time surveillance data collected without the need to enter cave sites using mobile phone-enabled high-frequency microphones for bat detection[51], validated and trained with reference acoustic calls using convolutional neural networks[52]. Identified bat species will be automatically linked with viral diversity data from EHA's host-pathogen database and SARSr-CoV data from DEFUSE to deliver high-risk pathogen lists, displayed as pathogen-centric, bat-centric, or map-centric views, with proactive alerts when critical information is received. All code modules will be available and documented on GitHub ([illegible]). <u>This technology will improve overall situational awareness of existing and novel infectious agents found in bats, allowing DoD personnel to quickly identify areas high spillover risk sites and rapidly deploy resources to respond to and mitigate their impact preemptively when necessary.</u>

SARSr-CoV QS detection, sequencing, and recovery. We will screen samples for SARSr-CoV nucleic acid using our pan-CoV consensus one-step hemi-nested RT-PCR assay targeting a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs[1,53], and specific assays for known SARSr-CoVs[2,21,33,34]. PCR products will be gel purified, sequenced and qPCR performed on SARSr-CoV-positive samples to determine viral load. Full-length genomes or S genes of all SARSr-CoVs will be high-throughput sequenced followed by genome walking[2,3,34]. We will analyze the S gene for its ability to bind human ACE2 by Biocore or virus entry assay. *<u>Synthesis of Chimeric Novel SARSr-CoV QS:</u>* We will commercially synthesize SARSr-CoV S glycoprotein genes, designed for insertion into SHC014 or WIV16 molecular clone backbones (88% and 97% S-protein identity to epidemic SARS-Urbani). These are BSL-3, <u>not select agents or subject to P3CO</u> (they use bat SARSr-CoV backbones which are exempt) and are pathogenic to hACE2 transgenic mice. Different backbone strains increase recovery of viable viruses identification of barriers for RNA recombination-mediated gene transfer between strains[34]. Recombinant viruses will be recovered in Vero cells, or in mouse cells over-expressing human, bat or civet ACE2 receptors to support cultivation of viruses with a weaker RBD-human ACE2 interface. *<u>Recovery of Full length SARSr-CoV:</u>* We will compile sequence/RNAseq data from a panel of closely related strains (<5% nucleotide variation) and compare full length genomes, scanning for unique SNPs representing sequencing errors[54-56]. Consensus candidates genomes will be synthesized commercially (e.g. BioBasic), using established techniques and genome-length RNA and electroporation to recover recombinant viruses[28,57].

Predicting strain-specific SARSr-CoV spillover risk. We will <u>combine detailed experimental characterization of QS_0 at our test cave sites with state-of-the-art genotype-phenotype Bayesian network models.</u> This will enable us to predict the jump probability of future QS that emerge with unique genetic recombinations. Our models will be parameterized with experimental data from a series of assays on the S genes of bat SARSr-CoVs (Fig. 6, right), with experimental and modeling work flowing together in iterative steps. Our prior data will act as baseline to parameterize spillover risk modeling[11,12,19,58]. This will be supplemented by characterization of isolated viruses under DEFUSE (at WIV), approximately 15-20 bat SARSr-CoV spike proteins/year (at UNC, WIV), and >180 bat SARSr-CoV strains sequenced in our prior work and not yet examined for spillover potential. All experiments will be performed in triplicate <u>and data fed to models in real time</u>:



Experimental assays of SARSr-CoV QS jump potential (Fig. 6, right). *<u>Pre-screening via structural protein modeling, mutation identification, and pseudovirus assays:</u>* Viral entry is the major species restriction preventing spillover of SARSr-CoVs[29,58]. To select QS for further characterization we will first use structural modeling of SARSr-CoV S protein binding to ACE2 receptors[59,60]. Mutations in the RBD[29,58,61,62], and host protease proteolytic processing of the S glycoprotein[63-65], regulate SARSr-CoV cell entry and cross-species infectivity. Mismatches in the S-RBD-ACE2 molecules or S proteolytic processing will prevent cell entry of SARS-CoV[29,58] and QS with these mismatches will be deprioritized. Single amino acid variations could dramatically alter these phenotypes and we will evaluate the impact of low abundant, high consequence micro-variation in the RBD using RNAseq to identify low abundant QS variants encoding mutations relevant to ACE2 binding. We will conduct *in vitro* pseudovirus binding assays, using established techniques[2], and live virus binding assays (at WIV to prevent delays and unnecessary dissemination of viral cultures) for isolated strains. Initial model predictions based on these data inputs will be used to guide strain selection for further characterization. *<u>In vitro testing of chimeric viruses:</u>* All chimeric viruses will be sequence verified and evaluated for: i) ACE2 receptor usage across species *in vitro*, ii) growth in primary HAE, iii) sensitivity to broadly cross neutralizing human monoclonal antibodies that recognize unique epitopes in the RBD[66,67]. Should some isolates prove highly resistant to our mAB panel, we will evaluate cross neutralization against a limited number of human SARS-CoV serum samples from the Toronto outbreak. Chimeric viruses that encode novel S genes with spillover potential will be used to identify SARSr-CoV strains for recovery as full genome length viable viruses. *<u>In vivo pathogenesis:</u>* Groups of 10 animals will be infected intranasally with 1.0×10^4 PFU of each vSARSr-CoV, clinical signs (weight loss, respiratory function, mortality, etc.) followed for 6 days

p.i., and sacrificed at day 2 or 6 p.i. for virologic analysis, histopathology and immunohistochemistry of the lung and for 22-parameter complete blood count (CBC) and bronchiolar alveolar lavage (BAL). *Validation with full-length genome QS:* We will validate results from chimeric viruses by re-characterizing full-length genome versions, testing whether backbone genome sequence alters full length SARSr-CoV spillover potential. QS for full-genome characterization will be selected to reflect strain differences in antigenicity, receptor usage, growth in human cells and pathogenesis. We will test growth in primary HAE cultures and *in vivo* in hACE2 transgenic mice. We anticipate recovering ~3-5 full length genome viruses/yr. *Testing Synthetic Modifications:* We will synthesize QS with novel combinations of mutations to determine the effects of specific genetic traits and the jump potential of future and unknown recombinants. *RBD deletions:* Small deletions at specific sites in the SARSr-CoV RBD alter risk of human infection. We will analyze the functional consequences of these RBD deletions on SARSr-CoV hACE2 receptor usage, growth in HAE cultures and *in vivo* pathogenesis. First, we will delete these regions, sequentially and in combination, in SHC014 and SARS-CoV Urbani, anticipating that the introduction of deletions will prevent virus growth in Vero cells and HAE[58]. In parallel, we will evaluate whether RBD deletion repair restores the ability of low risk strains to use human ACE2 and grow in human cells. *S2 Proteolytic Cleavage and Glycosylation Sites:* After receptor binding, a variety of cell surface or endosomal proteases[64-71] cleave the SARS-CoV S glycoprotein causing massive changes in S structure [72] and activating fusion-mediated entry[64,73]. We will analyze all SARSr-CoV S gene sequences for appropriately conserved proteolytic cleavage sites in S2 and for the presence of potential furin cleavage sites[74,75]. SARSr-CoV S with mismatches in proteolytic cleavage sites can be activated by exogenous trypsin or cathepsin L. Where clear mismatches occur, we will introduce appropriate human-specific cleavage sites and evaluate growth potential in Vero cells and HAE cultures. In SARS-CoV, we will ablate several of these sites based on pseudotyped particle studies and evaluate the impact of select SARSr-CoV S changes on virus replication and pathogenesis. We will also review deep sequence data for low abundant high risk SARSr-CoV that encode functional proteolytic cleavage sites, and if so, introduce these changes into the appropriate high abundant, low risk parental strain. *N-linked glycosylation:* Some glycosylation events regulate SARS-CoV particle binding DC-SIGN/L-SIGN, alternative receptors for SARS-CoV entry into macrophages or monocytes[76,77]. Mutations that introduced two new N-linked glycosylation sites may have been involved in the emergence of human SARS-CoV from civet and raccoon dogs[77]. While the sites are absent from civet and raccoon dog strains and clade 2 SARSr-CoV, they are present in WIV1, WIV16 and SHC014, supporting a potential role for these sites in host jumping. To evaluate this, we will sequentially introduce clade 2 disrupting residues of SARS-CoV and SHC014 and evaluate virus growth in Vero cells, nonpermissive cells ectopically expressing DC-SIGN, and in human monocytes and macrophages anticipating reduced virus growth efficiency. We will introduce the clade I mutations that result in N-linked glycosylation in rs4237 RBD deletion repaired strains, evaluating virus growth efficiency in HAE, Vero cells, or nonpermissive cells ± ectopic DC-SIGN expression[77]. *In vivo*, we will evaluate pathogenesis in transgenic hACE2 mice. *Low abundance micro-variations:* We will structurally model and identify highly variable residue changes in the SARSr-CoV S RBD, use commercial gene blocks to introduce these changes singly and in combination into the S glycoprotein gene of the low risk, parental strain and test ACE2 receptor usage, growth in HAE and *in vivo* pathogenesis.

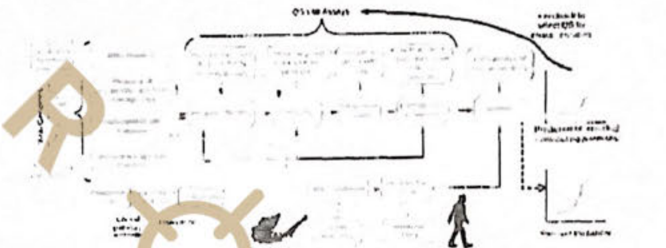


Fig. 7: A simplified directed graph of a Bayesian network model representing the causal relationships between input data, modeled processes, and outputs.

Network machine-learning to predict spillover potential of high-risk SARSr-CoV strains. We will use experimental data from above to build genotype-phenotype models of bat SARSr-CoV spillover potential. We will use Bayesian Network Models (BNM), fit via MCMC methods[78] to predict spillover risk based on bat SARSr-CoVs genotype data (presence of deletions in RBD, proteolytic binding and glycosylation sites etc.) and the ecological traits of hosts - integrating data on multiple, interacting processes and QS spillover potential to generate overall spillover probabilities. The Bayesian approach will allow us to update our models iteratively as new data is acquired, and use interim model predictions to guide which experiments to prioritize to maximize predictive ability[79]. We will control for experimental conditions (assays on live viral isolates, full-genome or synthetic chimeric viruses, and the molecular backbone of the latter). Traits will be used as inputs to BNM's causal graph, to predict latent variables representing interconnected processes that contribute to SARSr-CoV QS infection in new hosts : receptor binding, cell entry, immune system interaction, and intracellular growth, all measured by our lab assays. These, in turn will act as predictors for the ultimate outcomes of host pathogenesis and host jumping potential (Fig. 7). We will use published work on these genetic traits to put informative priors on strength and direction of interactions in the causal graph. We will use prior-knowledge model simulations to select target sequences from our sampling for characterization and genome-sequencing, to collect data that maximally enhances the predictive power of our model, and update these simulations iteratively throughout the experimental phase to continually guide QS selection. We will use regularizing priors to reduce over-fitting and select the most predictive variables in the final model.

Model validation using SARSr-CoV serology from previously-collected human samples and surveillance data. Active spillover of SARSr-CoV in our study region enables us to measure actual spillover risk to validate our models of QS jump potential. We will gather data on viral QS antibodies found in the local human population using LIPS assays on >2,000 previously-collected human sera (NIAID, Daszak PI) from people living close to our test cave sites in Yunnan Province, a sub-sample of which showed 2.7% seropositivity to bat SARSr-CoVs[13]. The IRB for

this work is current and covers proposed DEFUSE testing. We will design LIPS assays targeting high- and low-spillover risk SARSr-CoV QS, as done previously for SARSr-CoVs[13,80,81] and the novel SADS-CoV[14]. We will: 1) insert different high- and low-risk SARSr-CoV N genes into pREN-2 vector (LIPS vector), first assessing N gene similarity to determination their potential cross-reactivity in a LIPS assay; 2) determine LIPS assay specificity by producing polyclonal sera via injection of recombinant protein or attenuated virus into rabbits; 3) validate LIPS assays by incubating antigens with their respective positive serum samples and the antigen antibody complex eluted using protein A/G beads; 4) validate LIPS positive sera results by spike protein based LIPS and viral neutralization assay. As a confirmatory test, the positive samples from LIPS will be validated by virus neutralization assay. We will use these LIPS assays to test serum samples for presence of antibodies to high- and low-risk SARSr-CoV QS. We will validate predictions of jump potential and extend the BNMs to predict actual spillover probabilities by modeling bat-human contact rates with bats. We will use ecological data on bat hosts and human behavioral survey data collected previously from these individuals to estimate wildlife contact in predicting exposure measured by our LIPS assays.

Evolutionary modeling and simulation to predict potential strains. Our Bayesian network modeling will generate predictions of the spillover risk of QS sequences we identify. To examine risk associated with the total viral population, we will model and simulate evolutionary processes to identify likely viral QS that our sampling has not captured, and viral QS likely to arise in the future ("QS_x"). We will use a large dataset of S protein sequences and full-length genomes generated from prior work and DEFUSE fieldwork to estimate SARSr-CoV substitution rate and its genome-wide variation using coalescent and molecular clock models within a Bayesian MCMC framework[82]. We will estimate SARSr-CoV recombination rates at the cave population level using these data and Bayesian inference[83,84]. We will apply RDP[85], similarity plots, and bootscan to identify recombination breakpoints and hotspots within the SARSr-CoV genome as done previously[24], now extended to the full genome. Using these estimates we will simulate the evolution of the SARSr-CoV QS virome using a forward-time approach implemented in simulators that model specific RNA virus functions (e.g. VIRAPOPS[86]). We will predict the rate at which new combinations of genetic traits can spread in viral populations and compare recombination rates among caves and bat communities. Our forward-simulated results will provide a pool of likely unknown and future QS_x species. Using these and our SEM model for spillover risk, we will predict the QS_x most likely to arise *and* have spillover and pathogenic potential. We will use evolutionary simulation results to iteratively improve our Bayesian network model. The number of genetic traits with potential for prediction of pathogenicity is large, so we will perform variable reduction using tree-based clustering, treating highly co-occurring traits as joint clusters for prediction. We will generate these clusters from all SARSr-COV sequences from DEFUSE fieldwork and prior work. As trait clusters may be modified through recombination, we will use our forward-evolutionary modeling to predict how well trait clusters will be conserved, retaining only those unlikely to arise in unknown or QS_x genomes. This will enable a trade-off between increased predictive power based on current samples and generalizability to future strains that have not yet evolved.

Technical Area 2

Immune modulation approach to reducing bat SARSr-CoV spillover risk. Our work shows that the following unique immunological features of bats may explain their capacity to harbor high viral loads with minimal clinical signs: a) bats maintain constitutively high expression of IFNα that may respond to and restrict viral infection[17]; b) several interferon activation pathways are dampened, e.g. STING (a central cytosolic DNA-sensor molecule to induce interferon) dependent and TLR7 dependent pathways[16]; c) the NLRP3 dependent inflammasome pathway is dampened, and key inflammation response genes like AIM2 are not present in bats[87,88]. These traits may be due to bat immune-sensing pathway adaptation as a fitness cost of flight[15]. We hypothesize that bat virus replication will likely be restricted quickly by constitutively expressed IFNα in bats, resulting in lower B/T cell stimulation due to lower viral stimuli. Second, dampened interferon and inflammasome responses will result in lower cytokine responses that are required to trigger T/B cell dependent adaptive immunity (e.g. antibody response), ultimately resulting in suppression of viral replication and shedding. We and others have demonstrated proof-of-concept of this phenomenon: Experimental Marburg virus infection of Egyptian fruits bats, a natural reservoir host, resulted in widespread tissue distribution with low viral load, brief viremia, low seroconversion and a low antibody titer that waned quickly, suggesting no long-term protection is established[89-91]. Poor neutralizing antibody responses occur after experimental infection of bats with Tacaribe virus[92] and in our studies of experimental infection of bats with SARS-CoV (Wang, unpubl.). We also successfully showed that bat interferon can inhibit bat SARSr-CoVs[21]. We hypothesize that use of immune modulators that upregulate the naturally low innate immunity of bats to their viruses, will transiently suppress viral replication and shedding, reducing the host jump risk. We further hypothesize that because *Rhinolophus* bats are long-lived (20+ yrs in the wild), most bats in a population will have been exposed to a range of SARSr-CoV QS at our sites. Specifically targeting upregulation of their adaptive immunity (immune memory) to high-risk viral strains may lead to heightened clearance of high-risk strains. We will evaluate two immune modulation approaches to defuse spillover of SARSr-CoVs from bats to humans: 1) Broadscale Immune Boosting strategies (Wang, Duke-NUS): we will apply immune modulators like TLR-ligands, small molecule RIG-like receptor (RLR) agonists or bat interferon in live bats, to up-regulate their innate immunity and suppress viral replication and shedding; 2) Targeted Immune Boosting (Baric, UNC): the broadscale immune boosting approach will be applied in the presence of chimeric immunogens to activate immune memory in adult bats and boost clearance of high-risk SARSr-CoVs. We will use novel chimeric polyvalent recombinant S proteins in microparticle encapsidated gels for oral delivery and/or virus adjuvanted immune boosting strategies where chimeric recombinant SARSr-CoV S are expressed by raccoon poxvirus. Both lines of work will begin in Year 1 and run parallel, be assessed competitively for efficiency, cost, and scalability, and successful candidates from captive animal trials will be used in live bat trials at our test cave in Yunnan. The finding of low innate immunity across bats suggest that immune boosting could be broadly applicable to bat genera and viral families.

Broadscale immune boosting (Duke-NUS). We will work on the following key leads to identify the most effective approach to up-regulate innate immunity and suppress viral loads. *Toll-like receptor (TLR)/RIG-I Like Receptor (RLR) ligands:* Our work indicates a robust response in live bats to TLR-stimuli like polyI:C as measured by transcriptomics on spleen tissue (Fig. 8), liver, lung and lymph node, with matched proteomics to characterize immune activation *in vivo*. These activation profiles will be used to assess bat immune response to different stimuli and identify those which lower viral load in our experimental system at Duke-NUS (below).

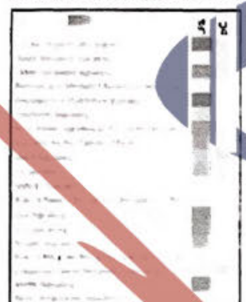


Fig. 8: Pathway analyses from Ingenuity Pathway Analysis (IPA) of whole spleen NGS after stimulation with either LPS or polyI:C. Z-score increase over control bats is indicated as per scale, and suggests strong activation of many pathways.

We will also stimulate the RIG-I pathway with 5'pppDSRNA, a mimetic of the natural RIG-I stimulant that will activate functional bat IFN production pathways, as shown in a mouse model that cleared SARS-CoV, IAV and HBV[18,19].

Universal bat interferon: We will design a conserved universal bat interferon protein sequence with artificial gene synthesis and produce recombinant protein by cleavable-affinity-tagged purification of supernatant from over-expressing bat cells, as used previously for recombinant *Pteropus alecto* IFNα[17,93] and CSF-1/IL-4. Utilization of a universal IFN for bats will overcome species-dependent response to the ligand, allowing the use of IFN throughout broad geographical and ecological environments and across many bat species. We have already produced recombinant non-universal, tagged, bat IFN that induce appropriate immune activation (Fig. 9). This ligand has been shown to reduce viral titers in humans, ferrets and mouse models intranasally and orally[18,19,94]. Interferon has been used clinically in humans as an effective countermeasure when antiviral drugs are unavailable, e.g. against filoviruses[20]. Interferon is known to be toxic, therefore we will carefully examine dose tolerance in bats and assess clinical effects of the treatment. We have shown that replication of SARSr-CoV is sensitive to IFN treatments[21]. The successful delivery, immune activation and outcome on the host will be characterized thoroughly to optimize rapid immune activation.

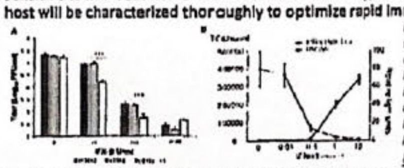


Fig. 9: Bat viruses are sensitive to IFN treatments. A) Recombinant bat SARS-related coronavirus WIV1 replication was inhibited by human IFN-6 in a dose dependent manner in Vero cells. B) Bat reovirus PRV1NB replication was inhibited by recombinant bat IFNα3 in a dose dependent manner in bat PakiT03 cells.

Boosting bat IFN by blocking bat-specific IFN negative regulators: Uniquely, bat IFNα is naturally constitutively expressed but cannot be induced to a high level, indicating a negative regulatory factor in the bat interferon production pathway[17,95]. We will use a *Pteropus alecto* CRISPRi library pool that we have created covering multiple RNA targets in every gene in the *P. alecto* genome (Wang, unpubl.

data). Genes affecting influenza replication in bat cells have already been identified using this library. Using CRISPRi we will identify negative regulator genes and screen for compounds targeting them to boost the inducibility of the IFN system in a shorter time-frame. Based on previous work[96-98], it is highly likely this will be a conserved pathway across all bats. *Activating dampened bat-specific innate immune pathways which include DNA-STING-dependent and TLR-dependent pathways:* We have shown that mutant bat STING or reconstitution of AIM2 and functional NLRP3 homologs restores antiviral functionality, suggesting these pathways are important in bat-viral coexistence. By identifying small molecules to directly activate pathways downstream of STING or TLR/RLRs, such as TBK1 activation, we will activate bat innate defense by interferons, promote viral clearance and, we hypothesize, significantly reduce viral load in bats. *Validation in a bat-mouse model.* Various CoVs show efficient infection and replication inside the human host but exhibit defective entry and replication using mouse as a host due in part to differences in DPP4 and ACE2 receptors.

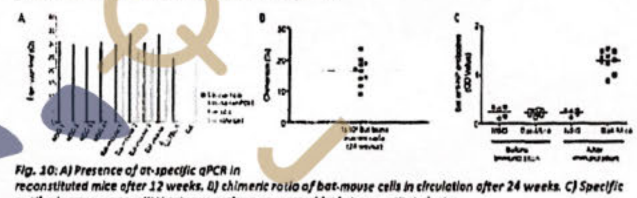


Fig. 10: A) Presence of at-specific qPCR in reconstituted mice after 12 weeks. B) chimeric ratio of bat-mouse cells in circulation after 24 weeks. C) Specific antibody response to a KLH-tetanus antigen generated by bat-reconstituted mice.

We have shown efficient reconstitution of irradiated mice using bat bone marrow from multiple species, including *E. spelaea* (Fig. 10), including reconstitution of bat PBMC's in the mouse, presence of circulating bat cells and generation of bat-specific antibodies in mice incapable of producing an antibody response. This 'batified' mouse model can be utilized for both circulating infection of SARS-CoV (in the immune compartment only) and as a model for generating bat-specific antibodies against CoV proteins. Efficient validation of infection into bat cells will be used to validate the infectivity of the viruses and generation of bat antibodies will facilitate validation of the best proteins/peptide to elicit an effective immune response.

Targeted immune boosting (UNC). To boost targeted adaptive immunity (immune memory) in wild bats chronically exposed to circulating SARSr-CoV QS, we will inoculate with chimeric S glycoproteins in the presence of the broadscale immune boosting agonists above. We have developed novel group 2b SARSr-CoV chimeric S glycoproteins that encode neutralizing domains from phylogenetically distant strains (e.g. Urbani, HKU3, BtCoV 279, ~25% diversity). The chimeric S programs efficient expression when introduced in the HKU3 backbone full length genome, and elicits protective immunity against multiple group 2b strains. We will develop robust expression systems for SARSr-CoV chimeric S using ectopic expression *in vitro*. We will work with Dr. Ainslie (UNC-Pharmacy) who has developed novel microparticle delivery systems and dry powders for aerosol release that encapsidate recombinant proteins and adjuvants (innate immune agonists) that we will use for parallel broadscale immune boosting strategies